A DETAILED STUDY OF THE FIELD DISTRIBUTION OF STRAINS OF CLOVER NODULE BACTERIA.

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(One Text-figure.)

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Introduction.

Although the difference in nitrogen fixing ability of different rhizobial strains is well known, and this property shows considerable specificity in relation to the nature of the testing host, little information appears to be available on the uniformity or otherwise of strains from within a smaller or larger area in the field.

An earlier detailed study (Hughes and Vincent, 1942), using antigenic properties as a guide to strain identity, has shown that isolations from the same species on the one farm can yield many distinct strains and that different nodules on the same plant are often inhabited by distinct serological types.

The present paper reports the results of similar and more extended studies, chiefly using the pattern of effectiveness with four testing clovers as criterion of strain identity. At the same time, serological data have been obtained for a large part of the isolated strains. Attention has also been given to possible relationships between the nature of the field host and behaviour with testing host, and the extent to which grouping within the four testing hosts appears to be valid.

For the present purpose, involving the simultaneous testing of a large number of cultures, an agar tube method is convenient and permits adequate differentiation between effective and ineffective strains.

Experimental.

Source of cultures.

Isolates were obtained in the usual way from clover plants collected according to the following schedule:

Series.	*Locality.*	*Field Host.*	*Year*
1[1]	Tichborne,[2] 4 sub-localities and paired large and small nodules.	T.G.	1947
2[1]	Parkes to Coonabarabran, 5 sub-localities and paired large and small nodules.	T.G.	1947
3	Tichborne[2] sub-locality 1.	T.G.	1948
4	Tichborne,[2] single additional sub-locality.	T.S.(*a*) T.R.(*a*) T.G.(*b*) T.T.(*b*)	1948

[1] Serological data available.
[2] At "Woodbine", the property of Mr. C. Watson.
(*a*) From plants sown as sterilised seed.
(*b*) From plants sown as sterilised seed and from naturally occurring plants.
T.G. = *Trifolium glomeratum* L.; T.S. = *Trifolium subterraneum* L.; T.T. = *Trifolium tomentosum* L.; T.R. = *Trifolium repens* L.

It will be observed that in series 1–3 the field host has been restricted to the one species. The soil at Tichborne is classified as red brown earth, and is relatively acid (pH approx. 5). Along the Parkes–Coonabarabran route soils are mainly red brown earths, with occasional patches of darker soils of higher alkalinity (e.g., soil from sublocality 5 was of this type). No detailed soil surveys have been made of this area.

Testing method.

Commercial seed of four clover species (*Trifolium glomeratum*, *T. subterraneum*, *T. pratense* L., and *T. repens*), sterilized with 1/500 mercuric chloride, germinated aseptically after the fifth washing and inoculated with the appropriate rhizobial strain, was planted at the top of an agar medium having the following composition:

$CaHPO_4$ 1 gm.; K_2HPO_4 0·2 gm.; $MgSO_4.7H_2O$ 0·2 gm.; NaCl 0·2 gm.; $FeCl_3$ 0·1 gm.; agar 8 gms.; water 1 litre. pH was adjusted to 6·5 with sodium hydroxide. In some experiments a nitrate control tube was included, containing 0·05% KNO_3.

Complete bacteriological control was aimed at throughout and only one out of eighty tubes used as uninoculated controls in many different experiments was found to be contaminated by rhizobium: mould and actinomycete development was consistently observed in a small number of cases and some tubes had to be discarded because of bad infection. In later experiments previously tested effective and ineffective strains were included for comparison.

A series of isolates was tested at the one time, provision being made for the analysis of block effect on replication and the position effect within the tube in relation to light source. Interaction terms were examined by comparison with error variance associated with the individual plant, using the *F* test. Estimates of effectiveness were based on the mean of single plant wet weights, known to be highly correlated with nitrogen content and much simpler to determine, transformed to logarithms to allow greater homogeneity of errors.

Criterion of effectiveness.

An improvement of 1·5 times the mean growth of control plants was selected as the mean level of effective strains. To equalize the chances of wrongly classing a mean either way, averages showing an improvement at least $0·5 \times \log 1·5 + \log$ control mean were classed as effective (E), the others being regarded as not different from the control and therefore, ineffective (I). The probability of the Type I (and Type II) errors is determined from the t value (one tail) using the interaction term (blocks × organisms) variance to determine the standard error of the mean deviation.

Classification of strains.

In the effectiveness tests these are classed according to the combinations of gradings assigned to each testing host. Serological groupings are arrived at in the usual way (Hughes and Vincent, 1942) according to the detailed flagellar and somatic reactions. In the present case more antisera were available than in the earlier paper.

Results.

General review of effectiveness tests.

Log weight mean values for each isolate have been grouped in classes and set out in Text-figure 1 to provide a histogram pattern for each host tested with each series of strains.

Certain features are common to all hosts:

(1) Uninoculated controls for each host are fairly consistent in different experiments, except for subterranean clover in series 1, where larger sized seeds were used.

(2) Of the two check strains tested in series 1, 3 and 4, the ineffective strain 4/12S has been consistent in its behaviour; strain 3/2L has been always effective, but has varied to some extent in its more detailed performance between experiments.

(3) Plants provided with nitrate have also varied in the growth attained. With the small seeded varieties, it is likely that insufficient nitrate was provided to supply an excess of nitrogen during the whole of the experiment.

Probability of the Type I (and Type II) errors (approximate values tabulated below) varies considerably for the different hosts, but is fairly consistent between experiments:

Host.	*Series.* 1	2	3	4
Trifolium glomeratum	>25%	20%	12%	30%
T. subterraneum	5%	1·5%	12%	7%
T. pratense	12%	15%	17%	17%
T. repens	22%	<25%	27%	27%

In general, the variances are least for subterranean clover and highest for ball and white clovers, and this is reflected in the magnitude of the errors which are directly related to the variances.

GROUPING OF STRAINS ACCORDING TO EFFECTIVENESS PATTERNS.

A summary of effectiveness patterns observed in the localities investigated is provided in Table 1.

Parallel reactions on test plants.

Statistical tests have shown that there is a better than chance parallelism of behaviour between responses on the paired hosts, ball and subterranean clover; red and white clover, despite the 75% disagreement observed between reactions of the second pair in the 1948, Tichborne sublocality 1 isolates. Concurrence between the two groups is no better than chance. It is interesting to observe the high proportion of cultures effective on the four hosts. (Strain A, Table 1.)

TABLE 1.

Frequencies of Strains from Various Localities Grouped According to Effectiveness Behaviour on Four Hosts.

Strain	Testing Host.				Tichborne.								Parkes-Coonabarabran.						General Total.	Percentage	
					Sub-locality.						1947 Total.	Tich-borne Total.	Sub-locality.					P-C. Total.		Tich-borne.	P.C.
					1947.				1948.												
	T.G.	T.S.	T.P.	T.R.	4	3	2	1	1	5			1	2	3	4	5				
A	E	E	E	E	3	7	10	7	7	60	27	94	7	2	2			11	105	49	26
B	E	E	E	I	11	4	4	2		11	21	32	1		2	1	3	7	39	17	17
C	E	E	I	E	1	3	3		21	8	7	36	1	3		3	1	8	44	19	19
D	E	E	I	I	4	3		1		1	8	9	1	3	5	2	4	15	24	5	36
E	E	I	E	E	1	2		1		1	4	5							5	3	0
F	E	I	I	E			1				1	1							1	<1	0
G	E	I	E	I	1		1				2	2							2	1	0
H	E	I	I	I	1	2		2			5	5							5	3	0
I	I	E	E	E			1				1	1							1	<1	0
J	I	E	I	E		1					1	1							1	<1	0
K	I	E	E	I							0	0							0	0	0
L	I	E	I	I	1						1	1							1	<1	0
M	I	I	E	E				1			1	1		1				1	2	<1	<1
N	I	I	E	I							0	0							0	0	0
O	I	I	I	E							0	0							0	0	0
P	I	I	I	I	1	1				1	2	3							3	2	0
	Totals ..											191						42	233		

E = effective reaction. I = ineffective reaction.

Results for a single block are quoted for Tichborne 1947 series tested on *T. glomeratum*. A marked difference in reaction was shown in the second block (where the testing conditions were somewhat different from all other tests) which would give a high proportion of ineffective on ball clover, a result not found elsewhere.

Testing hosts: *Trifoilum glomeratum* = T.G.; *T. subterraneum* = T.S.; *T. pratense* = T.P.; *T. repens* = T.R.

Comparison of major localities.

On the 1947 results, Tichborne shows a significantly greater dispersion of strains over the effectiveness patterns; the Parkes–Coonabarabran series, on the other hand, is practically restricted to patterns A–D, D being represented more than at Tichborne. However, the results for Tichborne in sublocality 1/1948, and the same sublocality in 1947, are markedly different and throw doubt on the validity of attributing the observed behaviour merely to localities.

Comparison of sublocalities in 1947.

It is not possible, on the basis of the full effectiveness patterns, to detect a sublocality effect, but if the comparison is restricted to reaction on white clover it will be seen that a significantly high proportion of ineffective strains was isolated from Tichborne sublocality 4 and Parkes–Coonabarabran sublocalities 3, 4 and 5.

Paired plant isolates.

Isolates with the same effectiveness patterns were treated as identical for the comparison of pairs from each plant examined. It has been found that the incidence of identical pairs (11/38 for Tichborne, and 7/20 for Parkes–Coonabarabran) was no greater than would be expected by chance, having regard to the distribution of effectiveness patterns in each series.

There is thus no evidence that invasion of a plant by one strain places any restriction on its invasion by a second strain.

Isolates from large and small nodules.

No more than random variation was observed in the incidence of effectiveness groups for isolates from large and small nodules. However it was noted from the wet weight yields in the 1947 Tichborne series that isolates from smaller nodules gave a significantly lower return on the homologous host than those from large nodules. Amongst the Parkes–Coonabarabran isolates, the reverse situation was encountered against white clover. However, those differences have not a very high order of significance and it would be difficult to generalize from them.

Strains collected by different field hosts.

On the basis of effectiveness patterns, isolates from *T. subterraneum* and *T. repens* (all from sterilized seed), *T. tomentosum* and *T. glomeratum* (including plants from sterilized seed and naturally growing specimens) showed no significant differences attributable to field host (Table 2).

TABLE 2.

Frequency of Different Effectiveness Patterns among Isolates from Four Field Hosts.

"Strain."	Effectiveness Pattern with Testing Host.				Field Host.				Total.
	T.G.	T.S.	T.P.	T.R.	T.S.	T.R.	T.T.	T.G.	
A	E	E	E	E	14	5	21	20	60
B	E	E	E	I	4	2	2	3	11
C	E	E	I	E	4		4		8
D	E	E	I	I				1	1
E	E	I	E	E				1	1
P	I	I	I	I				1	1
Total	..	..	.. ..	..	22	7	27	26	82

T.T. = *Trifolium tomentosum*. (Other abbreviations as given in Table 1.)

SEROLOGICAL TESTS.

Isolates from Tichborne 1947 and Parkes–Coonabarabran were tested for flagellar and somatic agglutination, against six antisera of known constitution. Nineteen different serological types were distinguished (Table 3).

TABLE 3.

Type Reactions with Six Antisera, Based on Flagellar and Somatic Agglutination, and their Frequency among 126 Isolates.

Serological Index.	Reaction with Serum.						Frequency.
	36	46	61	94	157	204	
Flagellar type a ..	H	–	H	H	–	H	59
Somatic type a_1 ..	+	–	–	–	–	–	11
,, ,, a_2 ..	–	+	–	–	–	–	13
,, ,, a_3 ..	–	–	+	–	–	–	6
,, ,, a_4 ..	–	–	+	+	–	–	11
,, ,, a_5 ..	–	–	–	+	–	–	2
,, ,, a_6 ..	–	–	–	–	–	+	10
,, ,, a_7 ..	–	–	–	–	–	–	6
Flagellar type b ..	–	H	–	–	H	–	45
Somatic type b_2 ..	–	+	–	–	–	–	7
,, ,, b_3 ..	–	–	+	–	–	–	5
,, ,, b_4 ..	–	–	+	+	–	–	20
,, ,, b_5 ..	–	–	–	+	–	–	4
,, ,, b_7 ..	–	–	–	–	–	–	3
,, ,, b_8 ..	–	+	+	–	–	–	6
Flagellar type c	–	–	–	–	–	–	19
Somatic type c_2 ..	–	+	–	–	–	–	2
,, ,, c_3 ..	–	–	+	–	–	–	3
,, ,, c_7 ..	–	–	–	–	–	–	13
,, ,, c_8 ..	–	+	+	–	–	–	1
Flagellar type d ..	h	h	h	h	h	h	1
Somatic type d_2 ..	–	+	–	–	–	–	1
Flagellar type e	H	–	H	h	H	h	2
Somatic type e_2	–	+	–	–	–	–	2

H, h, – indicate strong, weak and no flagellar reactions, respectively.
+, – indicate presence or absence of somatic reactions.

Comparison of major localities.

The distribution of some major serological types differed between the Tichborne and Parkes–Coonabarabran series (Table 4, cols. 6 and 7).

Comparison between sublocalities.

No heterogeneity could. be demonstrated amongst the sublocalities of Parkes–Coonabarabran, but was denfiite in the Tichborne sublocalities (Table 4, cols. 2–5).

Paired plant isolates.

In only one out of the four sublocalities of the Tichborne series was a greater than chance number of similar serological pairs encountered (5 observed as against 1·7 expected). No significant pairing was encountered among the Parkes–Coonabarabran isolates.

Isolates from large and small nodules.

There was a highly significant correlation between the size of the nodule and incidence of serological strains (Table 5), but before any general significance can be attached to this finding, it seems reasonable to correct for the effect of having taken sets of isolates from restricted areas where all similar strains might easily have arisen fairly recently from the one parent. For this reason figures of Table 5 are adjusted so that a particular strain is represented once only for a particular sublocality. There is now no better than a chance distribution of antigenic types in large and small nodules although it needs to be noted that the small number of cases available for the analysis makes significance difficult to demonstrate.

TABLE 4.

Distribution of the Most Frequent Serological Types in Tichborne Sublocalities and Parkes-Coonabarabran Major Locality.

Serological Type.	Tichborne Sublocality. 1	2	3	4	Tichborne Total.	Parkes-Coonabarabran.	General Total.
a_1	6	2	1	—	9	2	11
a_2	—	—	—	8	8	5	13
a_4	—	—	2	1	3	8	11
a_6	—	1	4	2	7	3	10
a residual ..	—	1	2	6	9	5	14
Total *a* ..	6	4	9	17	36	23	59
b_4	—	9	1	—	10	10	20
b residual ..	9	5	6	—	20	5	25
Total *b* ..	9	14	7	—	30	15	45
c_7	—	—	7	6	13	—	13
c residual ..	1	1	1	1	4	2	6
Total *c* ..	1	1	8	7	17	2	19
d	—	1	—	—	1	2	3
General total	16	20	24	24	84	42	126

TABLE 5.

Proportion of the Most Frequent Serological Types Found in Large and Small Nodules.

Serological Index.	Gross Figures.		With Sub-locality Correction.	
	Large Nodules.	Small Nodules.	Large Nodules.	Small Nodules.
a_1	9 (5·9)	2 (5·1)	5 (3·7)	1 (2·3)
a_2	12 (6·9)	1 (6·1)	5 (3·7)	1 (2·3)
a_4	8 (5·9)	3 (5·1)	5 (4·3)	2 (2·7)
a_6	7 (5·3)	3 (4·7)	5 (4·9)	3 (3·1)
b_4	7 (10·6)	13 (9·4)	4 (5·5)	5 (3·5)
c_7	1 (6·9)	12 (6·1)	1 (1·8)	2 (1·2)
Others	23 (25·5)	25 (22·5)	8 (9·1)	7 (5·9)
Totals ..	67 (67·0)	59 (59·0)	33 (33·0)	21 (21·0)

Figures in brackets represent expected values, calculated from cross totals.

Relationships between serological and effectiveness reactions.

Table 6 shows that serological characters might appear to be partially correlated with effectiveness patterns, but when account is taken of a possible locality effect even this imperfect correlation is reduced to an insignificant degree.

No correlation could be established between flagellar or somatic reactions separately and effectiveness patterns, when a correction for locality was applied in a similar manner.

TABLE 6.
Distribution of the Strains from Major Serological Groups in the Major Effectiveness Patterns.

Serological Index.	Gross Figures. Effectiveness Patterns.					With Sub-locality Corrections. Effectiveness Patterns.				
	A	B	C	D	Others.	A	B	C	D	Others.
a_1..	7 (3·4)	0 (2·5)	0 (1·3)	1 (2·1)	3 (1·7)	4 (2·1)	0 (1·7)	0 (1·1)	1 (1·6)	3 (1·6)
a_2..	1 (4·0)	8 (3·0)	0 (1·6)	2 (2·4)	2 (2·0)	1 (2·4)	4 (1·8)	0 (1·2)	2 (1·8)	2 (1·8)
a_4..	0 (3·4)	2 (2·5)	1 (1·3)	7 (2·1)	1 (1·7)	0 (2·1)	2 (1·7)	1 (1·1)	4 (1·6)	1 (1·6)
a_6	0 (3·1)	1 (2·3)	2 (1·2)	4 (1·9)	3 (1·5)	0 (2·6)	1 (2·1)	2 (1·3)	4 (2·0)	3 (2·0)
b_4..	12 (6·2)	3 (4·6)	2 (2·4)	2 (3·7)	1 (3·1)	5 (2·9)	2 (2·3)	2 (1·4)	1 (2·1)	1 (2·1)
c_7..	0 (3·7)	3 (2·7)	3 (1·5)	2 (2·2)	4 (1·9)	0 (2·6)	2 (2·1)	2 (1·3)	2 (2·0)	4 (2·0)
Others	18 (14·2)	11 (10·4)	7 (5·7)	5 (8·6)	5 (7·1)	14 (9·3)	8 (7·3)	5 (4·6)	4 (6·9)	4 (6·9)
Totals ..	38 (38·0)	28 (28·0)	15 (15·0)	23 (23·0)	19 (19·0)	24 (24·0)	19 (19·0)	12 (12·0)	18 (18·0)	18 (18·0)

Figures in brackets represent expected values.

DISCUSSION.

There is considerable evidence that most of the rhizobia isolated from clover in these wheat soils are capable of fixing nitrogen with *T. glomeratum*, which is the most common clover found growing naturally. The prevalence of rhizobia effective on the common natural host may have some relation to selective influences associated with it, but this is by no means certain.

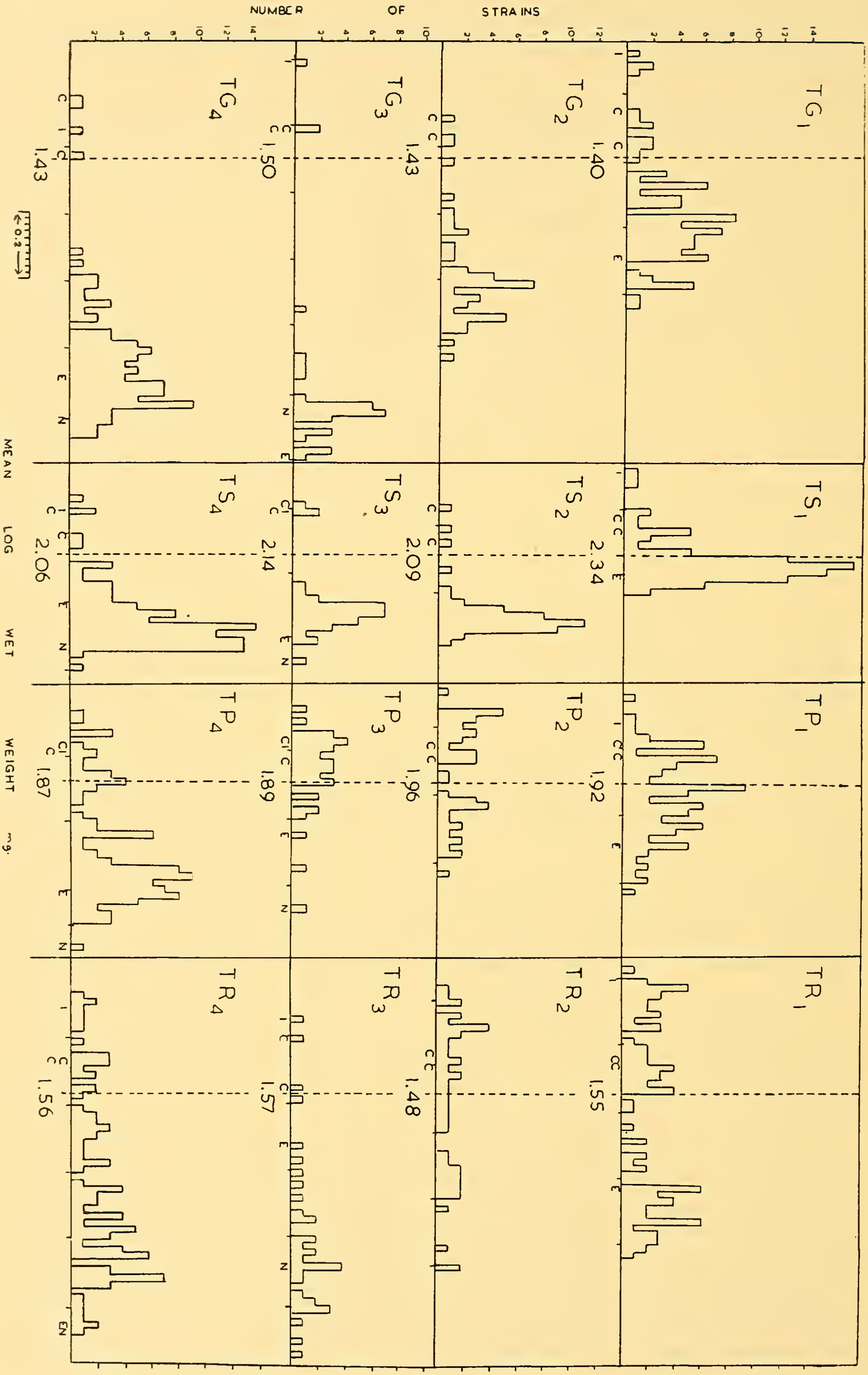
To some extent the observations of Strong (1937), on a degree of similarity of effectiveness behaviour on red and white clover, and dis-similarity on subterranean clover, has been confirmed (with ball clover being placed in the latter group, in accordance with the findings of Vincent, 1945), but the groups are by no means mutually exclusive, as has already been pointed out by Jensen and Vincent, 1941, and Vincent, 1945.

Relationships between effectiveness patterns and serological grouping are at best only partial, and are no more than would be expected in studying members of a localized field population probably having a fairly recent common origin. With similar serological types encountered in widespread localities, it can be considered that they are sufficiently removed in time from a common origin to have afforded greater opportunity for change in their behaviour on the host plant.

A change in the field population with time has been observed with regard to classification of strains based on (*a*) effectiveness patterns (Tichborne, sublocality 1, 1947, compared with 1948) and (*b*) serological types (1947 Tichborne data compared with those found earlier by Hughes and Vincent, 1942).

The occurrence of different serological types on the same plant was observed by Hughes and Vincent (1942); it is now assured that in general strains with different serological characters or different effectiveness patterns can be obtained from the one plant. Where identity between serological characters from two isolates from the one plant was observed in one locality, it probably indicates no more than that localization may have occurred within the small area (possibly in the rhizosphere). There is definitely no suggestion that the plant has any specific discriminating effect.

Difficulty was experienced in isolating strains with any particular effectiveness characteristics from larger or smaller nodules. This is probably due to the complications introduced by having nodules of different ages (cf. Nutman, 1946).



Text-figure 1.

The final picture that emerges from these investigations is that even within the immediate environs of the single plant there is a diverse rhizobial population characterized in the present instance by a general ability to fix nitrogen in association with the common clover of that area. Isolates from one or two nodules cannot give any real idea of the nature of the existing rhizobial population at any one time, much less its constitution on a second occasion. It would seem reasonable to expect that subterranean clover would be effectively nodulated if sown in an established ball clover area, but in the introduction of another clover species—such as red or white—into such an area, seed inoculation with a strain of known performance on these hosts (preferably also effective on ball and subterranean clovers) would be indicated. In such a case the ability of the introduced strain to establish and maintain itself in competition with local types would be of some importance even though initially the mass effect of inoculation would probably ensure nodulation predominantly by the introduced strain. Further studies in competition between strains are planned.

Summary.

Effectiveness patterns on four clover hosts and serological classification have been used to compare a large number of isolates from clovers growing in representative New South Wales wheat soils. Isolates were collected in such a way as to permit comparisons on the basis of locality, sublocality, the individual plant and on the basis of nodule size on the original field host.

Differences in effectiveness behaviour were observed between isolates from Tichborne and those from Parkes–Coonabarabran in 1947, and between cultures from one of the Tichborne sublocalities in two successive years. Serological types were heterogeneously distributed in different localities. No distinction could be made in effectiveness behaviour between ball clover isolates and isolates from other clover species sown in the Tichborne area.

A significant concurrence of effectiveness was noted between tests on ball and subterranean clovers, and white and red clovers using all the clover strains of *Rhizobium*, but agreement within each of these host groups was by no means complete.

No evidence was obtained of any restriction on the invasion of a plant by strains having different effectiveness patterns, but in one sublocality more identical serological types were obtained from paired nodules than could be expected by chance.

Isolates from small nodules showed somewhat lower ability to fix nitrogen on the homologous host than strains from large nodules, but results were inconsistent and little significance could be attributed to them. Certain serological types were found confined mainly to a particular nodule size but this observation could be mainly attributed to the localization of such strains to areas where they could have arisen from a single parent rhizobium.

No correlation between effectiveness patterns and serological behaviour could be observed above that attributable to the collection of isolates from small areas where certain rhizobial strains, probably of recent common parentage, were predominating.

The rhizobial population even within a restricted area is likely to be diverse and a few samples can give only a poor idea of its nature. The practice of seed inoculation may often be advisable to ensure nodulation with a suitable strain, but data on the strain's competitive ability would also be desirable.

Text-figure 1.

Frequencies of strains producing responses of host plants falling within specified mean logarithmic weights limits, for each series of four host species.

Abbreviations: T.G., *Trifolium glomeratum*; T.S., *T. subterraneum*; T.P., *T. pratense*; T.R., *T. repens*.

1, 2, 3, 4, series numbers for sets of strains.

I, ineffective strain, 4/12S; E, effective strain, 3/2L; C, uninoculated control (two tubes per series); N, 0·05% KNO_3.

Broken line represents the division between effective and ineffective responses.

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References.

HUGHES, D. Q., and VINCENT, J. M., 1942.—Serological Studies of the Root Nodule Bacteria. iii. Tests of Neighbouring Strains of the Same Species. PROC. LINN. SOC. N.S.W., lxvii, 142-152.

JENSEN, H. L., and VINCENT, J. M., 1941.—Specificity between Host Plant and Strain of *Rhizobium trifolii*. *Austr. J. Sci.*, 3 : 169.

NUTMAN, P. S., 1946.—Variation within Strains of Clover Nodule Bacteria in the Size of Nodules Produced and in the Effectivity of the Symbiosis. *J. Bact.*, 51 : 411-432.

STRONG, T. H., 1937.—The Influence of Host Plant Species in Relation to the Effectiveness of the Rhizobium of Clovers. *J. Coun. Sci. Indus. Res.*, 10 : 12-16.

VINCENT, J. M., 1945.—Host Specificity amongst Root Nodule Bacteria Isolated from Several Clover Species. *J. Austr. Inst. Agric. Sci.*, 11 : 121-127.